

C-CAM (Cell-CAM 105) is a calmodulin binding protein

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C-CAM (Cell-CAM 105) is a transmembrane cell adhesion molecule belonging to the immunoglobulin superfamily. It mediates intercellular adhesion of rat hepatocytes and occurs in various isoforms in several epithelia, vessel endothelia and leukocytes. We now report that purified liver C-CAM interacts specifically with calmodulin. Binding was observed both when ¹²⁵I-labeled C-CAM was used in a dot-blot assay and when ¹²⁵I-labeled calmodulin was used in a gel overlay assay. Experiments with protease-generated peptides indicated that calmodulin bound to the cytoplasmic domain of C-CAM. Analyses of whole liver membranes demonstrated that C-CAM is one of five major proteins that bind calmodulin in a calcium-dependent manner.

Cell adhesion molecule (CAM); Calmodulin; Cell adhesion; Transmembrane signalling

1. INTRODUCTION

Cell recognition and adhesion are mediated by cell adhesion molecules (CAMs) [1]. During the last decade a number of different CAMs have been identified, and molecular cloning and sequence analyses have revealed the existence of several CAM superfamilies [2]. It has been demonstrated that many of these molecules have important functions in morphogenetic processes, cellular invasion phenomena, homing reactions, and immunological events [1–3]. It is generally believed that CAMs not only mediate physical binding between cells, but also induce transmembrane signalling. However, the molecular mechanisms for CAM-triggered transmembrane signalling are not known. One approach for unravelling potential signalling pathways is to search for intracellular proteins that can bind to the cytoplasmic domains of transmembrane CAM proteins. We have used this strategy in our studies of C-CAM (Cell-CAM 105). C-CAM is a cell adhesion molecule with homophilic binding properties, occurring in liver, various epithelia, vessel endothelia, leukocytes and platelets [2,4–6]. It belongs to the CEA (carcinoembryonic antigen) gene family, which is a subfamily of the large immunoglobulin superfamily [2,4]. The liver isoforms are transmembrane proteins with a glycosylated 90–95 kDa

extracellular domain and cytoplasmic domains of either 71 or 10 amino acid residues [2,4], Edlund and Öbrink, unpublished). In the course of this work we found that a small protein could associate with C-CAM. The properties of this protein suggested that it might be calmodulin. Calmodulin, which is the major calcium binding protein in cells [7], regulates various enzymatic activities and can modulate the molecular interactions and behaviour of the cytoskeleton [8–10]. In this report we show by direct binding studies that C-CAM can bind calmodulin, and present some of the characteristics of this binding reaction.

2. MATERIALS AND METHODS

2.1. C-CAM, purification, labeling, proteolytic cleavage and phase partitioning

C-CAM was purified from rat liver and labeled with ¹²⁵I as previously described [11]. Purified C-CAM was cleaved by V8 protease or subtilisin essentially as described in [12]. Before digestion SDS was added to a final concentration of 0.5% and the solution was heated at 100°C for 2 min. The protein was then digested at 37°C with V8 protease (Boehringer, Mannheim, Germany; 50 µg/ml) for 30 min, or with subtilisin (Sigma Chemical Co., MO, USA; 40 µg/ml) for 5 min. Proteolysis was stopped by addition of PMSF (10 mM). The hydrophobic nature of C-CAM and the proteolytically derived peptides was assessed by partitioning into the detergent and aqueous phases of a Triton X-114 solution [13]. Since SDS interferes with the partition the SDS concentration was lowered by first diluting the samples with 2% Triton X-114 and then concentrating in Centricon microconcentrators (Amicon). The samples (100 µl) were then mixed with 2% Triton X-114 in TBS (100 µl) and incubated on ice for 30 min. They were then layered on a cushion of 6% sucrose in 1.5 ml Eppendorf tubes and incubated at 30°C for 3 min before centrifugation in an Eppendorf centrifuge at 6,000 rpm for 60 min. The resulting water, detergent and sucrose phases were analyzed by SDS-PAGE.

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2.2. Polyacrylamide gel electrophoresis and immunoblotting

Proteins were analyzed under non-reducing or reducing (10 mM DTT and boiling for 3 min) conditions on 10 or 14% polyacrylamide gels in the presence of SDS according to Laemmli [14] with the modification described in Hubbard and Lazarides [15]. The gels were dried and exposed to X-ray film with an intensifying screen. Immunoblotting was performed according to Burnette [16] using antibodies against C-CAM and 125 I-labeled protein A (Pharmacia-LKB, Uppsala, Sweden) as previously described [11].

2.3. Dot-blot assay

Proteins were spotted onto nitrocellulose filters (Schleicher and Schüll, 0.22 μ m) and dried using a hairdryer. Excess binding sites were blocked by incubating the filters for 30 min at 37°C in 5% BSA, TBS (0.15 M NaCl, 10 mM Tris-HCl, pH 7.5). The filters were then incubated with 125 I-labelled protein (1×10^6 cpm/ml) in TBS, 2 mM CaCl_2 , 5% BSA at room temperature for 1 h. Thereafter the filters were washed twice with TBS, twice with TBS containing 0.05% Triton X-100 and twice with TBS. The dried nitrocellulose filters were exposed to X-ray film (Amersham, Hyperfilm MP) with an intensifying screen.

2.4. Gel overlay assay

Calmodulin (from bovine testes, Pharmacia-LKB, Uppsala, Sweden) was labeled with 125 I by the lactoperoxidase method and used in a gel overlay assay as described by Curlin et al. [17]. Briefly, C-CAM was subjected to polyacrylamide gel electrophoresis in SDS, and the gels were fixed in isopropanol and acetic acid. After several washes in 0.2 M NaCl, 1 mM CaCl_2 , 50 mM Tris-HCl, pH 7.6, the gels were incubated with 125 I-calmodulin for 18 h (20×10^6 cpm) in the same solution containing either 1 mM CaCl_2 or 1 mM EGTA. The gels were washed, dried and exposed to X-ray film with an intensifying screen.

2.5. Isolation of plasma membranes

Plasma membranes were isolated from rat liver as previously described [11], and solubilized in 75 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 2 mM PMSF, 0.5% Triton X-100, 10 mM imidazole, pH 7.3. The suspension was centrifuged for 5 min in an Eppendorf microcentrifuge which resulted in a soluble supernatant fraction (S) and an insoluble cytoskeletal fraction (CK). The cytoskeletal fraction was resuspended in the solubilizing buffer to the original volume. The two fractions were analyzed by SDS-PAGE, calmodulin gel overlay assay, and immunoblotting.

3. RESULTS

3.1. Calmodulin binding to intact C-CAM

Fig. 1 shows a dot-blot analysis where calmodulin, C-CAM and BSA were spotted on a nitrocellulose filter which then was incubated with 125 I-labelled C-CAM. C-CAM bound to calmodulin and to itself, but not to BSA. We also investigated the binding of 125 I-labeled calmodulin to unlabeled C-CAM by gel overlay assay (Fig. 2). Both unreduced and mildly reduced C-CAM bound 125 I-calmodulin. However, when C-CAM was both reduced and boiled it lost its ability to bind 125 I-calmodulin. The binding was Ca^{2+} -dependent. When EGTA was included in the incubation buffer, the binding was completely abolished (Fig. 2B). None of the marker proteins, except phosphorylase *b* which is known to bind calmodulin [18], bound 125 I-calmodulin. Inclusion of trifluoperazine or chlorpromazine in the incubation buffer completely inhibited binding of 125 I-calmodulin to C-CAM (data not shown).

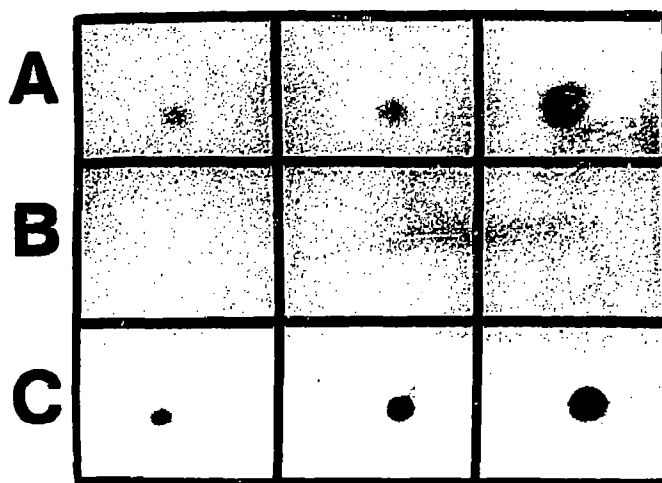


Fig. 1. Dot-blot analysis of binding between calmodulin and C-CAM. Different amounts of proteins were spotted onto a nitrocellulose filter which then was incubated for 1 h with 125 I-labelled C-CAM (1×10^6 cpm/ml) dissolved in TBS, 2 mM CaCl_2 , 5% BSA. The autoradiogram shows: in row A, calmodulin, from left to right 0.5, 1.0 and 2.5 μ g, respectively; in row B, BSA, from left to right 5, 10 and 25 μ g, respectively; in row C, C-CAM, from left to right 0.14, 0.28 and 0.7 μ g, respectively.

3.2. Calmodulin binding to peptides derived from C-CAM

In order to analyze which part of C-CAM interacts with calmodulin, binding experiments were performed with peptides derived from C-CAM by limited V8 or subtilisin cleavage. The V8 protease generated peptides with apparent molecular weights of 90, 56, 51, 45–48, 34, 24, 20 and 13–16 kDa; subtilisin generated peptides with apparent molecular weights of 84–88, 65–70, 37–39, 32 and 24 kDa (data not shown). Gel overlay analysis showed that 125 I-calmodulin only bound to the 84–88 kDa and 65–70 kDa peptides generated by subtilisin (Fig. 3A, lanes 3 and 4). None of the peptides generated by V8 protease bound calmodulin (Fig. 3A, lanes 1 and 2).

The hydrophobicity of the C-CAM peptides was analyzed by phase partition experiments in Triton X-114. Earlier experiments have demonstrated that intact C-CAM partitions exclusively into the detergent phase [5]. The 90 kDa V8-derived peptide partitioned into the water phase, whereas the 13–16 kDa V8-derived peptides partitioned into the detergent phase (Fig. 3B). Of the subtilisin-derived peptides the 84–88 kDa peptides and the 65–70 kDa peptides partitioned exclusively into the detergent phase, whereas none of the smaller peptides could be detected in this experiment (Fig. 3C). These experiments suggest that the V8-derived 90 kDa peptide contains most of the N-terminal extracellular portion of C-CAM, whereas the V8-derived 13–16 kDa peptides contain the transmembrane domain and most of the C-terminal cytoplasmic portion of C-CAM. The subtilisin-derived 84–88 and 65–70 kDa peptides should

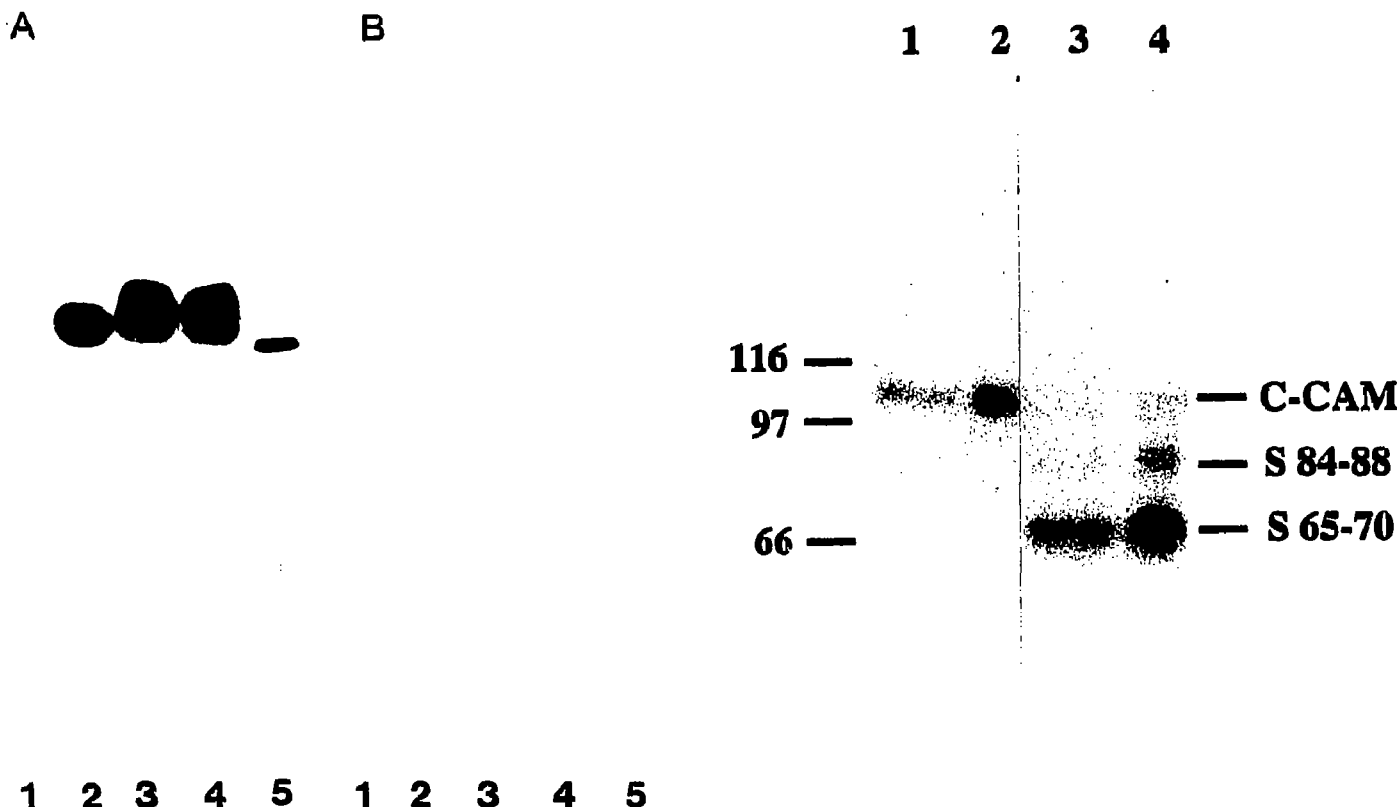


Fig. 2. Calmodulin gel overlay assay of purified C-CAM. C-CAM was subjected to SDS-PAGE and the gel was then incubated with [125 I]calmodulin (1×10^6 cpm/ml) in 50 mM Tris-HCl, pH 7.6, 0.2 M NaCl containing either 1 mM CaCl_2 (A) or 1 mM EGTA (B). Lane 1, C-CAM, 0.5 μg reduced and boiled; lane 2, C-CAM, 0.5 μg , unreduced and boiled; lane 3, C-CAM, 0.5 μg , reduced not boiled; lane 4, C-CAM, 0.5 μg , unreduced not boiled; lane 5, molecular weight markers (myosin, beta-galactosidase, phosphorylase *b*, BSA, ovalbumin, carbonic anhydrase). The positive protein in (A), lane 5, is phosphorylase *b*, which is known to bind calmodulin.

contain at least the transmembrane domain, a substantial portion of the extracellular domain, and probably some or all of the cytoplasmic domain.

Taken together, these experiments indicate that calmodulin binds to the cytoplasmic portion of C-CAM.

3.3. Calmodulin binding to liver plasma membrane proteins

To examine whether calmodulin binding to C-CAM is specific and not a hydrophobic interaction with membrane proteins in general, we analyzed the calmodulin binding pattern of liver plasma membranes. Coomassie blue staining showed that both the soluble and the insoluble (cytoskeletal) membrane fractions contained a complex pattern of polypeptides (Fig. 4A). A protein with a molecular weight of 105 kDa present in the soluble fraction bound [125 I]calmodulin under non-reducing conditions (Fig. 4B). Immunoblot analysis demonstrated that this protein migrated at the same position as C-CAM (Fig. 4D). Immunoprecipitation of the soluble fraction with anti C-CAM antibodies and analysis

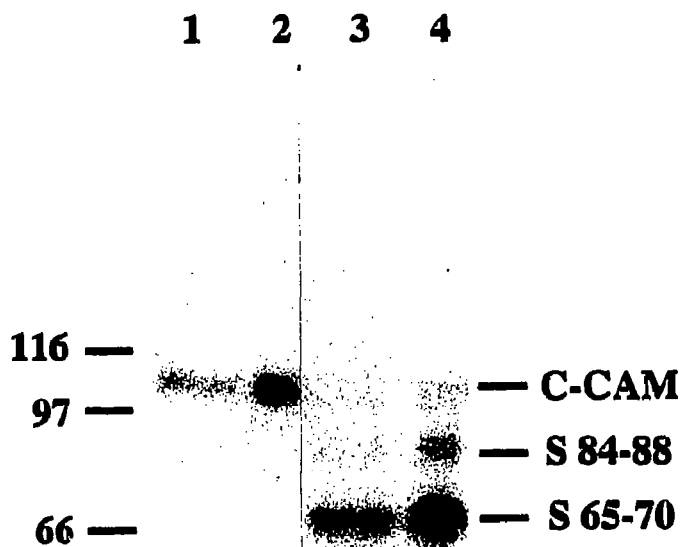


Fig. 3A.

Fig. 3. Calmodulin binding and phase partitioning of proteolytically derived C-CAM peptides. (A) Unlabeled C-CAM was digested with V8 protease or subtilisin, electrophoresed under reducing or non-reducing conditions on a 10% polyacrylamide gel, and analyzed by [125 I]calmodulin gel overlay assay in the presence of 1 mM CaCl_2 . Lane 1, V8 protease, reducing conditions; lane 2, V8 protease, non-reducing conditions; lane 3, subtilisin, reducing conditions; lane 4, subtilisin, non-reducing conditions. (B) [125 I]-labelled C-CAM was digested with V8 protease and subjected to partitioning in a Triton X-114:water phase system. The phases were analyzed by electrophoresis under reducing conditions on a 14% polyacrylamide gel and autoradiography. Lane 1, water phase; lane 2, sucrose phase; lane 3, detergent phase. (C) [125 I]-labelled C-CAM was digested with subtilisin and subjected to partitioning in a Triton X-114:water phase system. The phases were analyzed by electrophoresis under reducing conditions on a 10% polyacrylamide gel and autoradiography. Lane 1, water phase; lane 2, sucrose phase; lane 3, detergent phase.

by calmodulin gel overlay assay proved that this 105 kDa protein contained C-CAM (data not shown). Two large proteins with apparent molecular weights of 140 and 240 kDa, as well as two smaller proteins, also bound calmodulin. The inclusion of EGTA in the incubation buffer completely abolished the binding of calmodulin to all proteins, except to the 30 kDa protein, which still bound calmodulin but to a lower extent. The binding of calmodulin to C-CAM in the solubilized membranes was Ca^{2+} -dependent and sensitive to reduction.

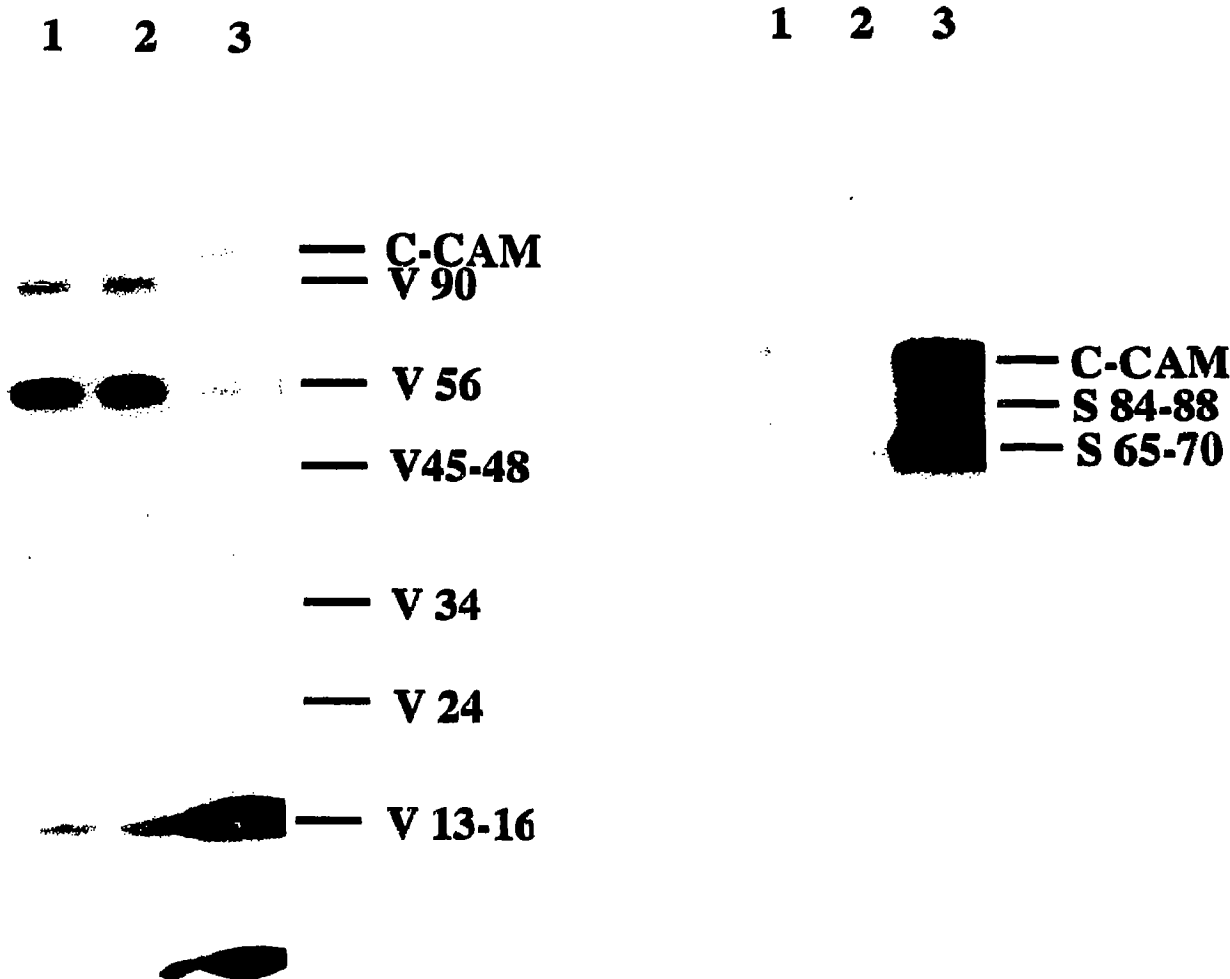


Fig. 3B.

Fig. 3C.

4. DISCUSSION

The present results show that C-CAM can bind calmodulin. This interaction seems to be specific since in a complex pattern of proteins in a liver membrane fraction only a few proteins, including C-CAM, bound calmodulin. Another fact pointing to a specific binding is that irreversibly denatured C-CAM did not bind calmodulin. Moreover, inclusion of trifluoperazine or chlorpromazine in the gel overlay completely inhibited binding of calmodulin to C-CAM. As for many other calmodulin binding proteins the binding to C-CAM required calcium ions.

Calmodulin is an intracellular protein. Thus, for the observed interaction between C-CAM and calmodulin to be of physiological significance, the binding site for calmodulin must be located to the cytoplasmic domain of C-CAM. The calmodulin binding pattern of the proteolytically derived peptides clearly indicated that this is the case. No binding was observed to the V8-derived 90 kDa peptide, which contains most of the extracellu-

lar portion of C-CAM, but a significant binding occurred to the subtilisin-derived 84-88 and 65-70 kDa peptides, which contain the transmembrane domain and most likely the cytoplasmic domain of C-CAM. However, the transmembrane domain did not seem to bind calmodulin since no binding was observed to the 13-16 kDa V8-derived peptides. The reason why no calmodulin binding peptides were recovered after V8 digestion most likely reflects cleavage of sequences important for calmodulin binding. Indeed, the large isoform of liver C-CAM has a V8-sensitive cleavage site in the cytoplasmic domain, between amino acid residues 510 and 511 [19]. Work is now in progress to produce recombinant proteins corresponding to the cytoplasmic domains of C-CAM for further studies of the calmodulin binding.

Calmodulin is a regulatory subunit of a number of cellular enzymes and interacts with components of the cytoskeleton [7-10]. Furthermore, several cell surface-associated proteins have been described to be calmodulin binding. These include the 110 kDa myosin I in

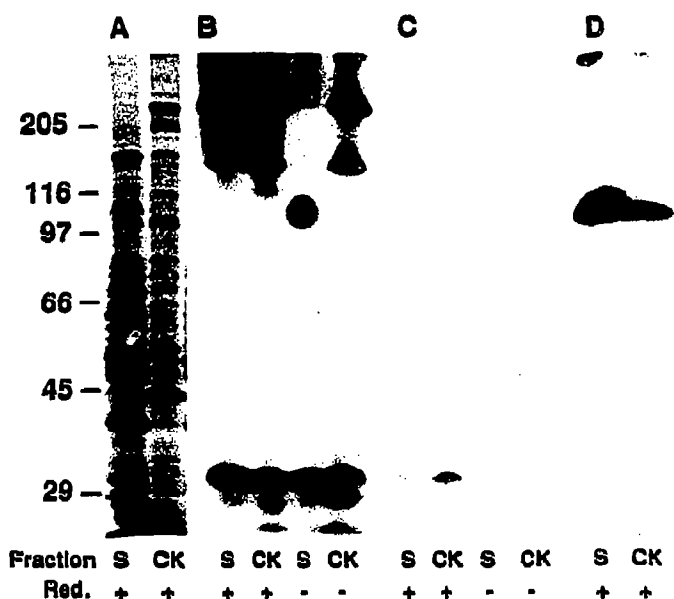


Fig. 4. Calmodulin gel overlay assay of liver plasma membranes. Isolated plasma membranes from liver were solubilized in 0.5% Triton X-100 and separated into a cytoskeletal (CK) and a soluble (S) fraction. The fractions were analyzed on a 10% SDS polyacrylamide gel, and either stained with Coomassie brilliant blue (A) or subjected to [125 I]calmodulin gel overlay assay in the presence of 1 mM CaCl_2 (B) or 1 mM EGTA (C) as described in the legend to Fig. 2. The two fractions were also analyzed by immunoblotting with anti-C-CAM antibodies and [125 I]protein A (D). Samples were analyzed both under reducing (+) and non-reducing (-) conditions as indicated in the figure.

intestinal epithelial cells [20], adducin in erythrocytes [9], and a 108/112 kDa protein in lymphocytes [21]. Calmodulin binding has also been observed to spectrin [8] and caldesmon [10]. Thus, it seems likely that the Ca^{2+} -calmodulin complex could regulate the organization and/or function of membrane-associated proteins. The analysis of the liver membranes showed that C-CAM is one of the major calmodulin-binding proteins in these membranes. We also observed calmodulin binding to two other proteins that were identical in size with spectrin (240 kDa) and caldesmon (140 kDa).

The functional significance of the interaction between C-CAM and calmodulin is presently not known. One possibility is that binding of calmodulin to C-CAM might affect the homophilic binding activity of C-CAM, thereby regulating the cell-cell adhesive activity of the cell surface. Another possibility is that homophilic binding between C-CAM molecules on adjacent cell surfaces

might alter the binding state of calmodulin to C-CAM. This might allow for cell adhesion-mediated control of the activity of the cytoskeleton and/or cell surface-associated enzymes. These two possibilities do not exclude each other but could be different co-existing parts of a system involved in a mutual transmembrane information flow between the interior and the exterior of communicating cells. Work is now in progress to test these hypotheses.

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